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*At*MYB61, an R2R3-MYB Transcription Factor Controlling Stomatal Aperture in *Arabidopsis thaliana*

Yun-Kuan Liang,[1,3] Christian Dubos,[2,3,4] Ian C. Dodd,[1] Geoffrey H. Holroyd,[1] Alistair M. Hetherington,[1] and Malcolm M. Campbell[2,*]

[1]Biology Department
Lancaster Environment Centre
University of Lancaster
Lancaster LA1 4YQ
United Kingdom

[2]Department of Botany
University of Toronto
25 Willcocks Street
Toronto, Ontario M5S 3B2
Canada

Summary

Stomata, dynamic pores found on the surfaces of plant leaves, control water loss from the plant and regulate the uptake of CO_2 for photosynthesis [1]. Stomatal aperture is controlled by the two guard cells that surround the stomatal pore [1]. When the two guard cells are fully turgid, the pore gapes open, whereas turgor loss results in stomatal closure. In order to set the most appropriate stomatal aperture for the prevailing environmental conditions, guard cells respond to multiple internal and external signals [2]. Although much is known about guard-cell signaling pathways [2–9], rather little is known about how changes in gene expression are involved in the control of stomatal aperture [10]. We show here that *AtMYB61* (*At1g09540*), a gene encoding a member of the *Arabidopsis thaliana* R2R3-MYB family of transcription factors, is specifically expressed in guard cells in a manner consistent with involvement in the control of stomatal aperture. Gain-of-function and loss-of-function mutant analyses reveal that *AtMYB61* expression is both sufficient and necessary to bring about reductions in stomatal aperture with consequent effects on gas exchange. Taken together, our data provide evidence that *AtMYB61* encodes the first transcription factor implicated in the closure of stomata.

Results and Discussion

Recently we found that misexpression of *AtMYB61*, which encodes an R2R3-MYB transcription factor, was both necessary and sufficient to explain aspects of the phenotype of the *Arabidopsis thaliana* mutant *de-etiolated3* (*det3*) [11]. Because the *det3* mutant had impaired stomatal function [12], we hypothesized that proper regulation of *AtMYB61* activity might be necessary for proper stomatal function. As a first step in testing this hypothesis, *AtMYB61* expression was tracked in transgenic plants harboring a transgene comprised of a translational fusion between the *AtMYB61* gene and a reporter gene encoding β-glucuronidase or Green Fluorescent Protein (GFP). Previously, *AtMYB61* expression was observed in developing vasculature and developing seeds [11, 13]. Expression in both of these tissues was transient—expression was only observed in particular cell types at specific stages of development [11, 13]. Detailed examination of the localization of GFP expression controlled by *AtMYB61* regulatory sequences revealed that *AtMYB61* was also expressed in guard cells (Figure 1A). In contrast with the transient expression observed previously [11, 13], *AtMYB61* expression in guard cells was observed throughout the lifetime of the plant. The localization of *At*MYB61 uniquely in guard cells raises the possibility that *At*MYB61 functions as a transcriptional regulator of stomatal function.

Infrared thermography can be used as a proxy indicator of stomatal function because plants with stomata that are more closed lose less thermal energy by evaporative cooling and, therefore, register as being warmer by thermography [14]. Conversely, plants whose stomata are more open should be cooler [14]. Consequently, infrared thermography was used to compare thermal energy emission in gain-of-function mutants (*MYB61OE*), loss-of-function mutants (*myb61*), and wild-type (WT) plants (Figure 2A). *MYB61OE* plants were generated by constitutive overexpression of the *AtMYB61* coding sequence under the control of a tandem duplication of the Cauliflower Mosaic Virus 35S promoter. The *myb61* loss-of-function mutants were identified from collections of insertionally mutagenized *A. thaliana* plants. Thermography revealed that *MYB61OE* plants were approximately 0.5°C warmer than WT plants, whereas *myb61* plants were approximately 0.5°C cooler than WT plants (Figure 2A). The findings with thermography suggest that constitutive *AtMYB61* expression results in more-closed stomata and that loss of *AtMYB61* activity results in more-open stomata.

Examination of stomatal aperture in epidermal peels of *A. thaliana* leaves showed that, relative to WT plants, *MYB61OE* plants had smaller stomatal apertures; in contrast, *myb61* mutants had larger stomatal apertures (Figure 2). These findings are consistent with the hypothesis that *AtMYB61* is both necessary and sufficient to partially close stomata. The role of *AtMYB61* in regulating stomatal aperture appeared to be independent of ABA, a well-established modulator of stomatal aperture (Figure 2). As in WT plants, stomatal closure in *AtMYB61* gain-of-function and loss-of-function mutants was responsive to increasing concentrations of ABA. These data are in contrast to the situation in well-known ABA-signaling mutants, such as *ost1*, which exhibit marked reductions in stomatal sensitivity to applied ABA [15]. The fact that *AtMYB61* mutants re-

*Correspondence: campbell@botany.utoronto.ca
[3]These authors contributed equally to this work.
[4]Present address: Laboratoire de Biologie des Semences, UMR 204 INRA/INA-PG, Département de Biologie Végétale, Centre de recherche de Versailles-Grignon, INRA, RD 10 Route de Saint-Cyr, 78026 Versailles cedex, France.

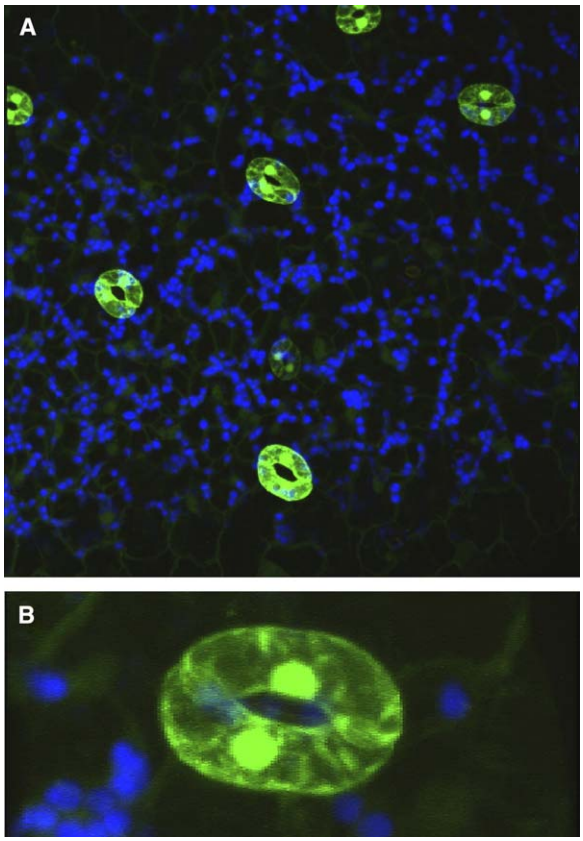

Figure 1. *AtMYB61* Is Specifically Expressed in Guard Cells

(A) Visualization of 61PN::GFP expression in the epidermis of *A. thaliana* leaves by confocal microscopy. Expression of Green Fluorescent Protein (false colored in green) was limited to the guard cells in the epidermis. Blue bodies correspond to the autofluorescence from chloroplasts. Seedlings were grown from seed for 28 days prior to analysis by confocal scanning laser microscopy. Seedlings were mounted on a microscope slide and examined with a Zeiss LSM 510 confocal laser scanning microscope according to published protocols [30].

(B) Higher magnification of a pair of guard cells.

mained responsive to ABA suggests that the pathway through which *AtMYB61* regulates stomatal aperture is distinct from the signaling pathway involving ABA.

Stomatal aperture impinges on whole-plant physiological processes, including the uptake of CO_2 for photosynthesis and loss of water vapor for evaporative cooling [1]. Consequently, mutants with altered stomatal function should differ from WT plants with respect to stomatal conductance (g_s). The gain-of-function *MYB61OE* mutants had a decreased g_s relative to WT plants (Figure 2C), which is consistent with these plants having an average decrease in stomatal aperture. Conversely, *myb61* mutants had an increased g_s relative to WT plants, consistent with these plants having an average increase in stomatal aperture. The relative differences in g_s between WT plants and the mutants were retained regardless of whether the plants were grown under conditions of normal or low relative humidity (Figure 2C). The mutant and WT plants were all clearly responsive to changes in evaporative demand and showed a decrease in g_s Nevertheless, relative to WT plants, *MYB61OE* plants still had the lowest g_s, and *myb61* mutants had the highest g_s. This shows that the response mediated by *AtMYB61* is retained independently of atmospheric humidity. Thus, *AtMYB61* does not appear to impinge upon ABA- or evaporative-demand-mediated control of stomatal aperture, raising the possibility that it is involved in another pathway that modulates stomatal aperture.

Stomatal aperture is well known to vary over diurnal cycles [16]. Stomata tend to be open during the day and tend to be closed at night. In wild-type plants, one can observe this trend simply by shifting light-adapted plants to dark conditions (Figure 3A). If *AtMYB61* activity controls stomatal aperture, the reduction in stomatal aperture when light-adapted plants are moved into the dark should be altered in *AtMYB61* mutants. Consistent with this hypothesis, *myb61* mutants did not close their stomata to as great an extent as wild-type plants in response to darkness. Again this is in contrast to the situation with the *ost1* mutant, which exhibits wild-type changes in stomatal aperture in response to a light-to-dark transition [15]. Interestingly, because light-adapted *MYB61OE* mutants had stomata that were already more closed than those in wild-type plants, they did not exhibit as dramatic a change in response to darkness as did wild-type plants. In fact, *MYB61OE* mutants and wild-type plants had the same stomatal aperture in the dark, suggesting that the *MYB61OE* mutants had achieved the maximum average closure. Importantly, WT plants, *myb61* mutants, and *MYB61OE* mutants were all significantly different ($p < 0.02$) in their dark-induced stomatal-closure response. Together, these observations suggest that *AtMYB61* is necessary and sufficient to account for a proportion of the changes in stomatal aperture in response to light-to-dark transitions, as might occur diurnally.

These data suggest that *At*MYB61 is involved in the dark-induced stomatal-closure signaling pathway. However, it is possible that *At*MYB61 plays a second, non-signaling role in stomatal closure. Under steady-state conditions, *myb61* stomata are more open and *MYB61OE* stomata more closed than wild-type stomata (Figures 2 and 3). Given that *AtMYB61* is required for the deposition of pectin in the testa of seeds [13], and that cell wall pectins have been shown to be important for stomatal movement [17], it is possible that the altered stomatal apertures in *MYB61OE* and *myb61* mutants may reflect altered structural properties of the guard-cell wall. Nevertheless, if this is the case, this role is in addition to the signaling role apparent in dark-induced closure but absent in guard-cell ABA and humidity signaling.

If *AtMYB61* is involved in the light-to-dark transition in stomatal aperture, its expression also might be expected to vary in a manner consistent with these trends. To test this hypothesis, diurnal changes in *AtMYB61* transcript abundance were examined with reverse transcriptase (RT) PCR on RNA extracted from leaves at a stage in development when *AtMYB61* expression was limited to stomata. On the basis of RT-PCR, *AtMYB61* transcript abundance was greatest in the middle of the dark phase and decreased notably in the light (Figure 3B). Consistent with these observa-

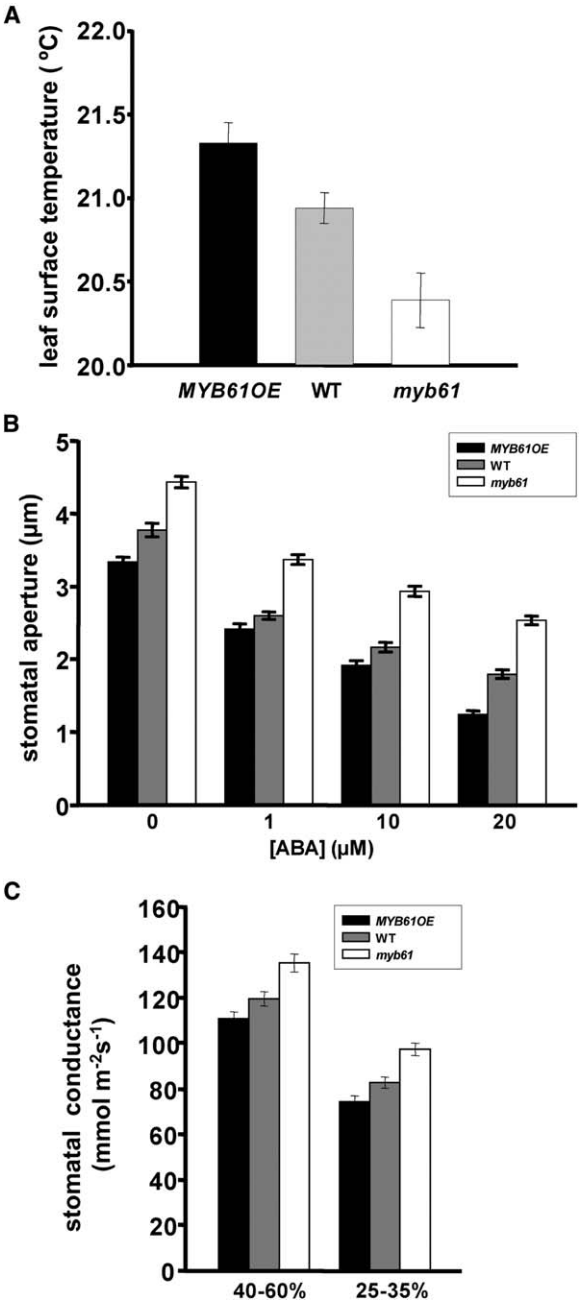

Figure 2. Manipulating the Expression of *AtMYB61* Alters Multiple Parameters Related to *A. thaliana* Stomatal Function

(A) Infrared thermography reveals that the leaves of *MYB61OE* are warmer and the leaves of *myb61* are cooler than those of WT. Leaf temperature was measured for 21- to 23-day-old plants (grown as described in [31]) with an Inframetrics. ThermaCam SC1000 focal plane array detector camera (3.4–5 μm) (Flir Systems) and analyzed with ThermaCAM Researcher 2001 software (Flir Systems). Plants were imaged under controlled-environment conditions (50%–70% RH, 21°C–23°C), and measurements were repeated on 3 successive days between hours 5 and 6 of the light period on all 3 days. Selected area temperature measurements are from ten leaves of five plants. The scale bars represent standard-error values.

(B) Stomatal aperture. The inhibition of stomatal opening on isolated epidermal strips from 5- to 6-week-old plants (grown as described in [31]) was investigated with the procedures described by Webb and Hetherington [31]. The figure shows the effect of ABA on stomatal aperture in *MYB61OE*, WT, and *myb61*, plants. The results are means ± SE of 120 stomata. The experiment was repeated three times. Two-way analysis of variance revealed that WT, *myb61*, and *MYB61OE* plants did not differ in their ABA response (i.e., genotype × ABA interaction was not significant, $p > 0.05$).

(C) Stomatal conductance. Plants were grown as per (C). One week before stomatal conductance was measured, the plants were moved to high (40%–60%) or low (25%–35%) relative humidity growth rooms (light intensity of 150 μmol $m^{-2} \cdot s^{-1}$). Abaxial stomatal conductance was measured on fully expanded leaves using a Delta T Instruments AP4 Porometer (Cambridge, UK). For each plant, conductance was measured on two separate leaves. The experiment was repeated three times, giving a total of 90 measurements for each line at 40%–60% RH and 30 measurements at 25%–35% RH. All measurements took place at light intensity of 150 μmol $m^{-2} \cdot s^{-1}$. All plants were analyzed between hours 5 and 6 of the light period. The results are means ± SE of ten measurements per line. Two-way analysis of variance revealed that the three genotypes did not differ in their humidity response (i.e., genotype × humidity interaction was not significant, $p > 0.05$).

tions, when plants expressing GFP under the control of *AtMYB61* regulatory sequences were exposed to continuous light, GFP was no longer detectable (Figure 3D). When these same plants were moved into the dark, GFP could be observed in the stomata again (Figure 3E). Notably, treatment of plants grown in the light for 72 hr with other stimuli known to induce stomatal closure did not result in expression of GFP. These treatments included spraying the plants with 100 μM ABA, watering plants with 400 mM NaCl, or depriving plants of water for 3 days (data not shown).

The results presented herein support the hypothesis that *AtMYB61* is both necessary and sufficient to decrease stomatal aperture. The fact that *AtMYB61* mutants still respond to ABA and that the responses to ABA and the mutations appear to regulate stomatal dynamics independently suggests that *AtMYB61* regulation of stomatal aperture acts in parallel with other stomatal-closure mechanisms that are invoked by the ABA signaling pathway (Figure 4). Similarly, *AtMYB61*-mediated regulation of stomatal aperture appears to act in parallel with mechanisms that close stomata in response to a reduction in relative humidity; such mechanisms almost certainly have an ABA component.

Based on the changes in the expression of *AtMYB61* in the light versus the dark, we propose that *AtMYB61* regulates stomatal aperture in response to diurnal signals (Figure 4). Given that *AtMYB61* expression is sucrose regulated [11, 13, 18], that there are significant diurnal fluctuations in sucrose concentration in guard cells [19–21], and that these diurnal fluctuations are predicted to function not only as osmoregulators but also as signals in guard-cell function [22, 23], it is appealing to suggest that sucrose may be the diurnal signal that modulates *AtMYB61* expression in guard cells. Future studies should interrogate the upstream regulatory machinery that modulates the timing and localization of *AtMYB61* expression relative to stomatal aperture.

Overexpression of *AtMYB61*, which decreases stomatal aperture, may prove a useful strategy for improving how efficiently plants that are grown under water-limiting conditions use water if such stomatal

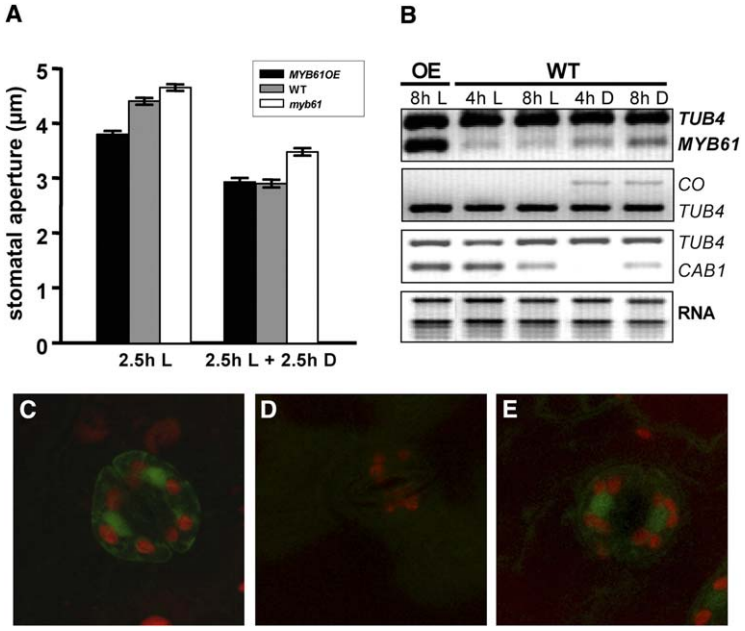

Figure 3. *AtMYB61* Expression is Correlated with Light/Dark Regulation of Stomatal Aperture

(A) Dark-induced promotion of stomatal closure is altered by *AtMYB61*. Freshly prepared epidermal peels of 5- to 6-week-old *MYB61OE*, *myb61*, and wild-type plants were prepared as per Figure 2. After 2.5 hr incubation in the light, half of peels were used for stomatal aperture measurement, and the remainder were transferred to dark conditions for a further 2.5 hr before measurements were made. The experiment was repeated four times and measurements were made between hours 4 and 6 of the light period on all days. Bars represent standard error (n = 210). Two-way analysis of variance revealed that the three genotypes differed significantly in their light response (i.e., genotype × light interaction was highly significant, $p < 0.0001$).

(B) RT-PCR analysis of *AtMYB61* transcript abundance. Five- to 6-week-old plants were grown in a 12 hr/12 hr light/dark cycle, and RNA was extracted from rosette leaves at 4 hr and 8 hr in the light period and 4 hr and 8 hr in the dark period. For collections in the dar, the only illumination provided was green safe light. RNA was extracted and analyzed by RT-PCR as previously described [11]. The lane designated *OE* was prepared with an RNA template derived from *MYB61OE* mutants at 8 hr in the light period. Two gene, *CONSTANS* (*CO*, *At5g15840*) and *CAB1* (*At1g29930*), were used as positive controls for diurnal fluctuations in transcript abundance, and *TUBULIN4* (*TUB4*, *At5g44340*) was used as a constitutively expressed control. RNA was extracted from leaves pooled from multiple plants. The lower panels show the ethidium-bromide-stained gel of template RNA, and one can see that RNA quantity and quality was equivalent for all samples. The images presented are representative of an experiment repeated three times.

(C) Visualization by confocal microscopy of 61PN::GFP Expression in the epidermis of *A. thaliana* leaves growing under long-day conditions (16 hr light/8 hr dark). Expression of Green Fluorescent Protein (false colored in green) was limited to the guard cells in the epidermis. Red bodies correspond to the autofluorescence from chloroplasts. The image is representative of >100 stomata visualized, in >10 plants, over an experiment repeated three times.

(D) As above except 72 hr after transfer to continuous light.

(E) As above except after 72 hr transfer to continuous light and then transfer to dark for 6 hr.

closure is non-limiting to photosynthesis. Given that *MYB61OE* plants also respond to ABA, a well-known drought signal, or to a decrease in relative humidity, these plants might enjoy the advantages of both constitutive and induced protection against water-limiting conditions. *AtMYB61* overexpression might be employed as a drought-resistance strategy for the growth of crop plants in arid regions.

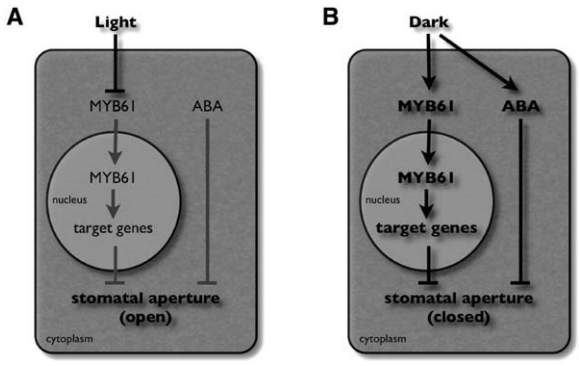

Figure 4. Model of the Relationship between *AtMYB61* Expression and the Control of Stomatal Aperture

(A) Schematic of guard cell in the light, with gray lines indicating absence or low levels of expression.

(B) Schematic of guard cell in the dark, with black lines indicating a high level of expression.

The link between the involvement of *AtMYB61* in lignification [11], testa mucilage formation [13], and changes in stomatal aperture is not obvious. It appears that *AtMYB61* regulates processes related to the acquisition and allocation of carbon, perhaps by balancing carbon supply with demand. The mechanisms underpinning how *AtMYB61* mediates these opposing processes warrant further investigation.

To the best of our knowledge, *AtMYB61* is the only example of a transcription factor that reduces stomatal aperture, although ectopic expression of the transcription factor ABI3 in guard cells affects stomatal movement [24]. Recently, *AtMYB60,* a gene encoding another R2R3-MYB family member, was found to increase stomatal aperture in response to diurnal cues (Cominelli et al., this issue [25]), underlining the importance of MYB transcription factors in this mode of regulation of stomatal function. The evolution of transcription-factor-encoding genes such as *AtMYB61* would have conferred a significant advantage to land plants as they colonized a terrestrial environment [26, 27]. Evolution of a transcriptional regulator that closes stomata, like *At*MYB61, would have enabled plants to reap the benefits of having stomata for carbon uptake while limiting the extent of water loss. Such genes continue to be of paramount importance today, not only because they

allow plants to adapt to changes in light, carbon, and water availability but because they shape the flux of carbon and water through entire ecosystems [1]. Thus, *AtMYB61* is an example of a key evolutionary innovation in the regulation of stomatal function and is likely to continue to impinge not only on physiological process but also on global-scale phenomena such as the carbon cycle.

Experimental Procedures

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* seeds (Col-0) were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC). Transgenic *Arabidopsis* plants overexpressing *AtMYB61* (overexpressor, OE) were generated as described previously [11]. *Arabidopsis* mutants that had insertional mutations in the coding sequence of *AtMYB61* (knockout, KO) were identical to those described previously [13]. KO allele-1 was isolated independently for this study and found to be allelic to that described previously (*myb61-1*) [13]; allele-2 [13] was generously provided by Dr. M.W. Bevan (John Innes Centre, Norwich, UK). The *AtMYB61::GFP* (*61PN::GFP*) fusion was constructed based on the *61PN::GUS* construct described previously [11]. The GUS coding sequence was replaced by the GFP coding sequence in order to generate the *61PN::GFP* construct, which was then stably transformed into *Arabidopsis* plants by the vacuum-infiltration-aided *Agrobacterium tumefaciens* transformation method ([28] as modified in [29]). *Arabidopsis* seeds were grown in soil comprising Levington's Universal soil and Vermiperl vermiculite (2:1). Plants were grown in temperature-controlled growth rooms at 22°C with an average light intensity of 130 μmol/m²s, at day lengths indicated in the figure captions.

Supplemental Data

Supplemental Data include two movies and are available with this article online at http://www.current-biology.com/cgi/content/full/15/13/1201/DC1/.

Acknowledgments

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